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The Role of Energetic Metabolism in the t-haplotype Transmission Ratio Distortion

Úloha energetického metabolismu při odchylce od Mendelovské dědičnosti v případě t-haplotypu u myší

Master´s Thesis

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Declaration:

I declare that I wrote this work myself and that I stated all information sources and literature I used. This work or its significant part was not submitted in order to obtain another or the same academic title.

Prague, 14. 8. 2017

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 14. 8. 2017

Bc. Hana Marvanová

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Abstract

When two alleles carried by a heterozygote are transmitted unequally to the zygote at the time of fertilization, transmission ratio distortion occurs. The best studied example of this phenomenon in mammals is t-haplotype in mice. The mouse t-haplotype is a selfish variant region on chromosome 17, in nature transmitted as a unit. Male mice homozygous for t haplotype are sterile, but heterozygotes transmit the t haplotype up to 99% of their progeny. This is believed to be caused by motility differences between sperm carrying the t haplotype and wild-type sperm from the same heterozygous male. The concrete mechanism of the postulated sperm competition in favour of t haplotype carrying sperm was so far not fully illuminated.

During this project, we worked with the hypothesis that the differences in sperm motility putatively responsible for transmission ratio distortion are triggered, at least in part, by metabolic causes.

Our results from ATP and mitochondrial membrane potential (MMP) comparison indeed suggest that there are metabolic dissimilarities in sperm from the different genotypes of t (t/t, t/+, +/+). Specifically, our data show that there is significantly less ATP in t/t sperm when compared to the other two genotypes. Likewise, sperm from t/t mice also seem to have lower MMP, suggesting that their mitochondria are less functional.

A t-haplotype targeted qPCR assay was established in order to test if there is a correlation between metabolic characteristics of sperm and its t-haplotype in heterozygous samples. Since the established method requires further optimization, we could not conclude with certainty if subpopulations of sperm with different MMP are enriched in a specific haplotype. At any rate, our preliminary data do suggest that this is indeed the case.

Key words: transmission ratio distortion, non-mendelian inheritance, t-haplotype, sperm metabolism

Abstrakt

Jsou-li dvě heterozygotem nesené alely přenášeny do další generace v nestejném poměru, nastává jev nazývaný odchylka od Mendelových pravidel. Nejlépe popsaným případem tohoto fenoménu mezi savci je t haplotyp u myší. T haplotyp je sobeckou variantou regionu na chromosomu 17, který se v přírodě zpravidla přenáší jako jednotka, tedy bez účasti rekombinace. Zatímco samci homozygotní pro t haplotyp jsou sterilní, heterozygoti přenáší t haplotyp až 99 % svých potomků.

Jak v současnosti věříme, tento jev je způsobován rozdíly v parametrech motility mezi spermiemi nesoucími t haplotyp a těmi nesoucími originální variantu tohoto regionu, jež obojí pochází od jednoho heterozygotního samce. Konkrétní mechanismus postulované kompetice, jejímž výsledkem je zvýhodnění spermií nesoucích t haplotyp, však zůstává doposud neobjasněn.

Pracovní hypotézou tohoto projektu je, že rozdíly v motilitě potenciálně odpovědné za odchylku od Mendelových pravidel dědičnosti jsou, aspoň z části, způsobovány metabolickými příčinami.

Naše výsledky srovnání hodnot ATP a mitochondriálního membránového potenciálu (MMP) vskutku naznačují, že mezi spermiemi samců porovnávaných genotypů (t/t, t/+ a +/+) existují jisté metabolické rozdíly. Konkrétněji, naše data poukazují na signifikantně nižší hodnoty ATP u spermií pocházejících od homozygotů t/t, než jaké byly naměřeny u dvou dalších testovaných genotypů. Stejně tak byly u spermií od homozygotů t/t zaznamenány signifikantně nižší hodnoty MMP, což naznačuje sníženou výkonnost jejich mitochondrií.

Abychom odhalili případnou korelaci mezi metabolickými charakteristikami a haplotypem spermie pocházející od heterozygota t/+, byla ustanovena qPCR analýza zacílená na t haplotyp. Vzhledem k tomu, že si tato metoda vyžaduje další optimalizaci, nebylo doposud možné vyvodit žádné závěry ohledně toho, jestli je subpopulace definovaná na základě hodnoty MMP z heterozygotního t/+ vzorku obohacena o specifický haplotyp. Nicméně, předběžná data tomu nasvědčují.

Klíčová slova: odchylka od Mendelových pravidel, nemendelovská dědičnost, metabolismus spermie

List of abbreviations

ATP	Adenosine triphosphate
CASA	Computer assisted sperm analysis
CCCP	carbonyl cyanide 3-chlorphenylhydrazone
DMSO	Dimethylsulfoxid
DTT	Dithiothreitol
FC	Flow cytometry
HLA	Human leukocyte antigen
JC-1	5´,6,6´-tetrachloro-1,1´,3,3´-tetraethylbenzimidazolylcarbocyanineiodide
KR medium	Krebs Ringer medium
MMP	mitochondrial membrane potential
NTC	non-template control
RBC	Red blood cell
SC	Standard curve
Tcd	t-complex distorter
Tcr	t-complex responder
wt	wild type
t/+	heterozygote for t haplotype
t/t	homozygote for t haplotype
+/+	wild type

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1. Introduction

It is a basic principle of genetics that each allele is transmitted from parent to offspring with a probability that is given by Mendel´s laws. According to his law of segregation, in diploid heterozygous organisms is the likelihood of spreading to the next generation equal for both variants of the trait.

However, there are known biological processes disrupting this balance of nature. Departures from Mendelian transmission proportions can be caused by several mechanisms, such as meiotic drive, gametic competition and embryo lethality (Huang et al., 2013).

Meiotic drive systems can be roughly divided into two categories: chromosomal and genic. Chromosomal drives may be caused by variances in structure or size of chromosomes that imply and advantage to the spindle apparatus and occurs in females (Lyttle, 1991).

Genic meiotic drive is represented by segregation distorters, which can also be described as “selfish” or “ultra-selfish DNA”. The later does not provide any advantage to their carriers, but induces self-transmission, by destruction of its allelic opponent (Crow & Dove, 1988; Orgel & Crick, 1980).

Both situations result in a phenomenon known as transmission ratio distortion (TRD). According to the definition of Mary F. Lyon, transmission ratio distortion are “*instances in which the two alleles carried by a heterozygote are transmitted unequally to the zygote at the time of fertilization*” (Lyon, 2003).

To identify such cases in nature, a drive element must not have reached fixation, and there must be genetic markers that enable the non Mendelian segregation ratios to be measured (Lyttle 1993).

The two best-studied and illuminated cases of genic drift in nature are the t-haplotype in mice and the SD system in *Drosophila melanogaster* (Lyttle 1993). Segregation distortion genes in drosophila cause dysfunction of wild type sperm (SD^{+}) during spermatogenesis, resulting in five to ten times bigger likelihood to be transmitted to the next generation from an heterozygote (Sandler et al., 1959). T haplotype in mouse

represents the most studied case of TRD in mammals and is the object of this master thesis.

As any TRD phenomenon is a genetic force, which affects allele frequency at the population as well as at the organismal level, it has implications on genetic diversity of the population over time (Huang et al., 2013). Therefore, studying a specific TRD phenomenon can contribute to increase the knowledge of genetics, both at population and developmental levels.

1.1. TRD in human

TRD mechanisms can be falsely interpreted as a linkage or association signal in family-based studies searching for linkage between a marker and a specific disease. The main criterion for tested group in this type of studies is the incidence of the disease in the family. Therefore, occurrence of TRD can be misinterpreted and should be kept in mind as a possibility. To positively identify cases of TRD, families (with offspring unselected for phenotype) should be screened for transmission patterns within two or three generations and genotyped (Huang et al., 2013).

The actual prevalence of TRD occasions in human remains so far largely unknown. Even in recorded cases, the mechanism of TRD is usually not fully enlightened. The following are just a few examples of the already described cases (reviewed by Huang et al., 2013).

One of detected cases of TRD in human is myotonic dystrophy, an autosomal dominant neuromuscular disease, which is caused by CTG-repeat expansion in Dystrophia myotonica protein kinase (DMPK) locus of chromosome 19q13.3. A preferential transmission of larger alleles (with more than 29 repeats) was found from females only (Chakraborty et al., 1996).

The other way around, preferential transmission of shorter alleles (with less CAG repeats) was observed in patients with Machado-Joseph disease, which is an autosomal dominant neurodegenerative disorder associated with CAG repeat expansion in the MJD1 gene located in the region 14q32.1. TRD was observed in female meiosis only, transmitting the shorter alleles in 57% of observed cases (Rubinsztein & Leggo, 1997).

TRD was also described in the context of Spinal muscular atrophy (SMA). This recessive neurodegenerative disorder is caused by loss or mutation of the telomeric survival motor

neuron gene SMN1. Transmission ratio of SMN1-deleted alleles was examined and resulted in confirmed TRD in favour of the SMN1 wild type alleles (Botta et al., 2005).

Another TRD event was recorded in the context of pericentric inversion in the region 1.p36.21-1q42.12 responsible for infertility and congenital anomalies (Honeywell et al., 2012).

There are as well TRD showing loci linked to autoimmunity functions located in the region of major histocompatibility complex on chromosome 6. As an example, evidence of TRD was found in CLIC1 gene, coding for a ubiquitous intracellular chloride-ion channel, in two of tested single nucleotide polymorphisms (CLIC1-2230 SNP, CLIC1+3712 SNP) and appeared to be paternal and offspring dependent (Hanchard et al., 2006).

Transmission ratio for most common HLA haplotypes was examined which resulted in finding that extended haplotype HLA-B8, DR3, SCO1, GLO2 was transmitted from males to 83% of the offspring (Awdeh et al., 1983).

Possible TRD loci in human were also reported in various cancer types, schizophrenia, cystic fibrosis and others (Huang et al., 2013; Zöllner et al., 2004).

It is also worth noting that many recessive lethal mutations with possible transmission distortion effect might be active in earliest stages of embryonic development even before pregnancy is detected.

A better understanding of these mechanisms in other examples in nature might enable us to detect and identify potential cases of transmission ratio distortion in human. Taking TRD phenomenon into consideration could potentially benefit many aspects of human genetics. It has a potential impact on practical aspects of human genetics such as correct interpretation of association study results (Huang et al., 2013) and for the genetics of fertility (Zöllner et al., 2004).

For better understanding TRD phenomenon in human, we can gain an inspiration in the most studied case of TRD in mammals, the mouse t-haplotype. *"To date, the mouse t-haplotype is among the best characterized and best understood of selfish genetic elements, at both the genetic an molecular levels"* (Herrmann & Bauer, 2012).

1.2. History of the t-haplotype

In early 1920´s, Nadine Dobrovolskaia-Zavadskaia had been interested in the question whether radiation effects could be transmitted hereditarily in mice. From this question raised one of the first successful genetic screens for developmental mutants in mice (Korzh & Grunwald, 2001). In that study, she observed and described short tailed phenotype in the offspring of X-ray irradiated mice and suggested a genetic cause (Willison & Lyon, 2000).

In 1927 Nadine Dobrovolskaia-Zavadskaia isolated and characterized a mutation affecting Brachyury T, a gene that regulates tail and axial development in the mouse (Dobrovoskaia-Zavadskaia & Kobozieff, 1932; reviewed in Korzh & Grunwald, 2001).A dominant allele of Brachyury marked by T was identified as the cause of short tail. Brachyury T in heterozygosity (T/+) results in mice with short tails. On the other hand, T in homozygosity (T/T) is lethal, resulting in embryos that lack notochord and posterior mesoderm (Chesley, 1932, 1935).

As a second, recessive t° was identified, inducing lethality in homozygosity (Gluecksohn-Schoenheimer, 1940). Although there was no abnormal phenotype observed in t°/+ mice, t° variant enhanced the effect of T in T/t° mice, resulting in complete taillessness (Dobrovoskaia-Zavadskaia & Kobozieff, 1932).

Since T and t° did not show any crossing over, they were considered alleles (Kobozieff, 1935). Although none of them damaged embryonic development in heterozygous state, they were considered part of a balanced system causing embryonic lethality (Dobrovoskaia-Zavadskaia & Kobozieff, 1932). That is, the interaction of two allelic genes, one dominant and one recessive, both lethal in homozygous condition, were responsible for the tailless condition when combined (Bennett, 1975).

There was found no indication of linkage with other mutant characters (as albinism or pink-eye) commonly examined at that time (Clark, 1934).Further breeding experiments were performed with the purpose of better understanding the interactions between the identified alleles. As a result of these crossings, four phenotypes were observed: TT abnormal embryos dying 11 days after fertilization, tailless T/t° mice, T/+ mice with short tail (defined as Brachy phenotype) and t°/+ normal mice (Chesley & Dunn, 1936) (Figure 1: Chesley, Dunn 1936; expected and observed phenotypes from experimental crossing).

A tailless × Brachy			
T/t^0		$T/+$	
T/T	T/t^0	$t^0/+$	$T/+$
dies	tailless	normal	Brachy

Figure 1: Chesley, Dunn 1936; expected and observed phenotypes from experimental crossing

Another remarkable observation coming from these crossings were significant departures from the expected transmission ratio linked to male mice. It was proven that +/t° males produce a significantly higher proportion of tailless progeny when tested by Brachy than do +/t° females. The same way males but not females of the genotype T/t° produced far more normal-tailed than short-tailed offspring when crossed with wild-type mice (Chesley & Dunn, 1936).

Moreover, different +/t° males appeared to give different proportions of tailless progeny (Chesley & Dunn, 1936). This observation suggested the existence of several recessive t-alleles, which was confirmed later (Dunn, 1955). Different t alleles evinced various embryonic lethal phenotypes in homozygous condition (reviewed in Bennett, 1975). The discovery of the group of t alleles led to the suggestion that these different recessive mutations might in fact be chromosomal rearrangements rather than true mutations (Dunn & Caspari, 1945). After this suggestion, wild populations were investigated and even more t allele versions were found and isolated. All of them produced taillessness when combined with T allele, and mice with normal tail when combined with wild type. When homozygous, some of them were lethal and others were viable and resulted in normal tail length. Moreover, they expressed various abnormal segregation ratios when the t allele was carried in the male (Dunn, 1955), with transmissions ranging from 75% to 97%, depending upon the concrete allele (Bennett, 1975). These distorted progeny ratios are identical whether the heterozygous male carries t next to wild type allele (t/+) or the dominant allele Brachyury (t/T) (Dunn, 1960).

Recessive t-alleles were then classified into three categories: lethal, semi-lethal and viable (reviewed in Bennett, 1975). A common feature of all discovered t alleles was their tail-modifying effect when combined with T (Bennett et al., 1969). Lethal alleles were subdivided into 6 different groups according to their effect on the developing embryo

(Yanagisawa et al., 1974). Semi-lethal alleles were defined as a class in which a proportion of homozygotes dies before birth, although some survive and become morphologically normal adults (Bennett et al., 1969). Variants classified as viable versions of t allele kept their enhancing effect on T, but did not cause death of developing embryo when in homozygosity (Yanagisawa et al., 1974).

Interestingly, male mice carrying two t alleles were sterile or semi-sterile, whereas females of the same genotypes were fertile (Bennett & Dunn, 1967). Male homozygous for any of the semi-lethal alleles and males carrying two different t alleles (compound homozygous) were always sterile. Importantly, it seems clear that t-associated sterility is not related with the number of spermatozoa entering the female tract, but to compromised sperm motility (Bennett & Dunn, 1967).

1.3. Molecular basis

The first speculations about the physical basis or these genetic defects led to ideas about deletions and inversions on chromosomal level, but the idea of t alleles as representation for chromosomal abnormalities did not survive for long (reviewed in Bennett, 1975). The existence of so many different t alleles with a modifying effect on the original T gene led to rejection of one pleiotropic locus idea and suggested that different "t alleles" resided at separate loci (reviewed in Herrmann & Bauer, 2012b).

Extensive breeding experiments of mouse tail mutants (including T) have proven their close location at the same chromosome (Dunn & Caspari, 1945). The reason why all the mentioned effect are commonly inherited as a unit was explained by observation of recombination suppression (Dunn & Caspari, 1945) which have been believed to be caused by differences in the chromatin structure (Dunn & Gluecksohn-Schoenheimer, 1950).

Later it was clarified, that a group of genes occupying a region of 30 to 40 Mb of the proximal part of chromosome 17 is responsible for effects originally associated to "t allele" (Silver, 1985).

This region of genes bounded by recombination suppression, currently known as t-complex, occurs in nature as a variant to its wild type form (Silver, 1985).

The original T gene, which pointed out the existence of its modifying “t alleles”, was also localised into this region. There were also another normal genes identified in this region, besides those which caused observed effects (Silver, 1985). Mary F. Lyon succeeded with isolation of the gene causing hair loss called tufted (tf), 8 cM distal to T on the wild type chromosome, which provided a tool for genetic dissection of the T locus (Lyon, 1959).

By using position of tf gene, Mary F. Lyon generated group of “t alleles” which were actually partial t haplotypes of the complete t-complex. She managed to do so by tracking rare recombination events between the wild type chromosome and the t containing chromosome in the region between tf and T genes (Lyon & Meredith, 1964a, 1964b).

This way, the region within the t-complex was dissected, which resulted in mapping of T and other factors responsible for lethality and sterility (LS). Additionally, the factor responsible for transmission ratio distortion (TRD) was narrowed to a more concrete region and called t-complex responder, aka Tcr (Lyon & Mason, 1977). It was proven that these factors can be separated but retain their genetic properties (Lyon & Mason, 1977).

While the inheritance of complete t-haplotype reaches full TRD effect up to 99% of the transmission cases, partial t haplotypes are always transmitted with lower level of transmission rate. The responder locus Tcr was identified as the crucial locus which must be present in heterozygosity for TRD to occur. When present solely, Tcr is transmitted with a ratio under 50%. Therefore, it was suggested that different genetic factors of t-complex are contributing to the Tcr causing the high transmission effect (Lyon, 1984). This suggestion was confirmed by finding other factors contributing to TRD afterwards (Hammerberg, 1982; Hammerberg, 1981). These new factors were called t-complex distorters (Tcds). In the absence of Tcr, the distorters expressed transmission according to expected Mendelian ratios. (Lyon, 1984).

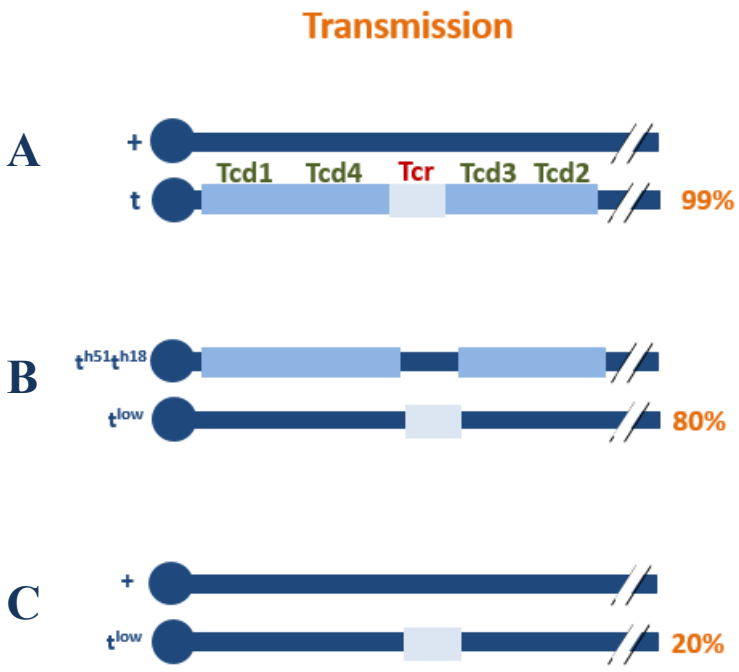

Figure 2: Schematic representation of a complete t haplotype (A) which is transmitted to 99% of the progeny; (B) distorters (Tcds) acting in trans on the responder (Tcr), which is transmitted in ratio higher than Mendelian; (c) the responder (Tcr) without distorters is transmitted at a low ratio

Mary F. Lyon subsequently postulated a model explaining the inheritance of the t-haplotype. The t-complex distorters (Tcds) exert their effect on the t-complex responder (Tcr) and increase the transmission of the responder bearing chromosome. The transmission rate of partial t-haplotype increases according to how many distorters are involved. The t-complex responder without involved distorters is transmitted in low ratios. But the distorters themselves do not induce TRD if Tcr is absent. The transmission rate of Tcr increases independently of the chromosome containing the distorters. Therefore, the distorters act in trans, while the responder in cis (Lyon & Zenthon, 1987; Lyon, 1986).

1.4. The structure of t-haplotype

It is now settled that t haplotype is a variant region of chromosome 17 spanning around 43 Mb or 20 cM (Lyon et al., 1988). This region occurs in two forms in nature –wild type (wt) and t-haplotype (Silver, 1985). Recombination suppression in this region arose from chromatin mismatches due to four large inversions within the region (Hammer et al., 1989; Herrmann et al., 1986). Tcr is located in the central part of the region. The

distorters were mapped to subregions within the inversions (Lyon, 1986). The inversions were termed In(17)1, In(17)2, In(17)3, and In(17)4. Originally, existence of three distorters (Tcd1, Tcd2, Tcd3) was postulated in the areas of these inversions (Lyon, 1984).

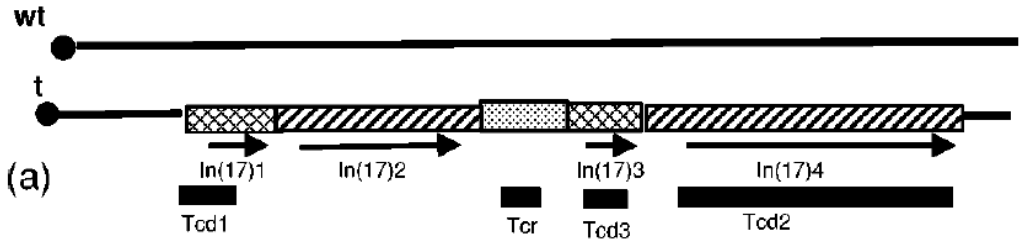

Figure 3: Representation of the wild type (wt) and t-haplotype (t) variants of the mouse chromosome 17. The t-haplotype contains 4 inversions marked In(17)1-In(17)4. The position of the genetic factors responsible for TRD mechanism (Tcr, Tcd1-3) is marked. Adopted from (Mary F Lyon, 2003)

1.5. Male sterility

Male mice are described as sterile if they do not produce progeny when kept with females of known fertility for a minimum of two months (Bryson, 1944). Male homozygous for t haplotype are sterile (Dunn, 1937).

According to the model of Mary F. Lyon, male sterility in mouse t-haplotype is due to the interaction of different sterility factors. Based on partial haplotypes studies, the sterility factors were suggested to be identical to the distorter genes (Tcds) (Lyon, 1986).

The distorter genes (Tcds) cause the transmission of the chromosome carrying the t form or the responder when heterozygous, and result in full or partial male sterility when homozygous (Lyon, 1986). It was also observed that different combinations of partial t haplotypes cause different levels of reduction in fertility or complete sterility (Lyon, 2003; Lyon, 1986; Silver, 1985).

The motility of the sperm cells from homozygotes for t-haplotype is described as non-progressive and they are reported to have reduced sperm-zona pellucida binding abilities (reiewed in Olds-Clarke, 1997; Olds-Clarke, 1983). This suggests that t-haplotype factors are affecting spermatozoa in some way.

Since the distortion and sterility effect are expected to be caused by the same group of factors, the ability of the t-haplotype to spread to the next generation can paradoxically lead to sterility.

1.6. Physiological basis of TRD

It raises a question, whether t haplotype, when in heterozygosity, can cause the degeneration of wild type chromosome bearing sperm cell at any point within spermatogenesis or later. It was proven that in t-carrying heterozygous males the chromosomes bearing either t-haplotype or wt form are distributed in a 1:1 ratio during metaphase I of meiosis. It implies that heterozygous males produce equal proportions of germ cells carrying the t-haplotype and germ cells not carrying the t-haplotype (Hammerberg & Klein, 1975).

Also, no evidence for specific spermatid degeneration was found. The amount and type of observed defects does not show any correlation with t-haplotype (Hillman & Nadijcka, 1978). The wt-form bearing spermatozoa from heterozygous males are not degraded during spermatogenesis or during the fertilization (L. M. Silver & Olds-Clarke, 1984).

This series of previously mentioned experiments suggests that TRD in t-carrying heterozygous males has a cause in competition of sperm for egg cell. To observe this competition, epidydimal sperm from t/+ heterozygotes and +/+ wild type homozygous males were mixed and used for artificial insemination into the uterus of T/+ females. The results showed that the t-carrying sperm cells from heterozygous males are not superior to sperm from +/+ wild type males. It points out a disadvantage of wild type variant carrying sperm cells from males heterozygous for t-haplotype (Olds-Clarke & Peitz, 1985).

This disadvantage causes, that in t/+ heterozygous males, spermatozoa not bearing the t-haplotype are defective in fertilization compared with t-bearing spermatozoa (Olds-Clarke, 1997; Olds-Clarke & Peitz, 1985).

While looking for the type of disadvantage affecting the spermatozoa not bearing the t-haplotype, a comparison of motility functions was performed.

There were indeed found differences in flagellar movement detected by comparing observation of sperm samples from homozygotes t/t, heterozygotes t/+ and wild type +/+

mice (Olds-Clarke & Johnson, 1993). These differences were lately confirmed by Computer assisted sperm motility analysis comparison of different genotypes of t (Amaral, unpublished data). Furthermore, two subpopulations in terms of motility parameters were observed in heterozygous t/+ sperm samples (Amaral, unpublished data).

Based on these observations, the molecular model of TRD mechanism was suggested, postulating that the distorter genes express products which impair sperm motility, and that Tcr provides resistance to this harmful effect only in t-sperm, leading to an advantage and to ratio distortion in favour of t sperm (Lyon, 1986).

1.7. Responder and distorters identification

Partial haplotypes enabled to track the genetic factors responsible for TRD till the level of chromosomal regions, but not to the level of single genes. The search of candidate genes for the distorters (Tcds) and the responder (Tcr) started.

1.7.1. The responder

By partial haplotypes studies, the position of the responder Tcr was narrowed to a region between inversions In(17)2 and In17(3) (Lyon, 2003).

In 1999 Bernhard G Herrmann succeed in the identification of the gene responsible for the effects attributed to the responder Tcr. He discovered a protein kinase, which he termed Sperm motility kinase (SMOK) coded by Smok gene. SMOK is a serine/threonine kinase that may be involved in sperm motility. The coding region in t haplotype for alternative protein $SMOK^{Tcr}$ is composed of the promoter and coding exon of Smok gene and part of the Rsk3 gene (as a variant called $Smok^{Tcr}$). The transcribed fusion protein $SMOK^{Tcr}$ is able to counterbalance the effects of the distorter proteins. The expression of both Smok and $Smok^{Tcr}$ has been detected only in testes, with $SMOK^{Tcr}$ as a dominant negative form of SMOK (Herrmann et al., 1999).

$Smok^{Tcr}$ transgenes were integrated to the mouse genome and a series of mouse lines with $Smok^{Tcr}$ transgene on one chromosome and partial t-haplotype on the other chromosome were established. It was proven that $Smok^{Tcr}$ is transmitted to the next generation with a higher than Mendelian ratio in presence of partial t haplotypes containing the distorter regions. When combined with wild type chromosome 17, $Smok^{Tcr}$ is transmitted in

proportion lower than Mendelian (Herrmann et al., 1999). By this experiment the identity of $Smok^{Tcr}$ as the responder Tcr was proven (reviewed in Herrmann & Bauer, 2012).

1.7.2. The distorters

Mary F. Lyon proposed the existence of three distorter factors. Candidate genes for these responders were searched for in the region of the inversions causing recombination suppression and defining the whole t-complex. Existence of Tcd4 and Tcd5 was suggested but not confirmed (Silver & Ramis, 1987).

Three criteria for distorter candidate genes were defined by the group of Bernhard G Herrmann. A potential distorter candidate must be located in the t complex region, has to show variability between t-haplotype and wild type variant and has to be expressed in the testes (Bauer et al., 2012; Herrmann & Bauer, 2012a).

Firstly, Tagap1 (T-cell activation Rho GTPase-activating protein) was mapped to the Tcd1 locus. Tagap1 encodes a GTPase-activating protein for Rho small G proteins. The t haplotype contains four copies of wild type Tagap1 with four alternative products. This t-haplotype version is called $Tagap1^{Tcd1a}$. Its role in TRD was confirmed by artificial overexpression and knock-out of Tagap1, which both changed the transmission ratios (Bauer et al., 2005).

Secondly, Fgd2 (faciogenital dysplasia 2) was isolated in the region of Tcd2. Fgd2 encodes a guanine nucleotide exchange factor acting on Rho small G proteins. The t allele of Fgd2 called $Fgd2^{Tcd2}$ is overexpressed in testis compared with wild type (Bauer et al., 2007).

Nme3 (Nucleoside diphosphate kinase gene) was identified in Tcd2 locus. Its t allele is called $Nme3^{Tcd2}$. The reduction in the Nme3 gene dosage increases the transmission rate of the t-haplotype (Bauer et al., 2012).

A common feature of those identified distorters is that they all act on Rho small GTPases. Rho small GTPases play a role in signal transduction and can therefore influence cell motility and cell polarity (Jaffe & Hall, 2005).

1.8. The molecular model of TRD

During spermatogenesis, the cells which are derived from the same spermatogonial cell develop connected in syncytium (Burgos & Fawcett, 1955). The cells are connected by intercellular bridges which permit the sharing of the cytoplasmic constituents. This type of sharing ensures a synchronous development of the haploid spermatids (Erickson, 1973). Therefore, genetically different haploid spermatids can be phenotypically equivalent (Braun et al., 1989).

The distorter products are indeed shared through intercellular bridges. However, Smok and the responder $Smok^{Tcr}$ represent an exception to this rule. The products of Smok and $Smok^{Tcr}$ are retained in the haploid sperm cells without sharing through intercellular bridges of the syncytium (Véron et al., 2009). In case of t haplotype heterozygosity half of the spermatids contain $SMOK^{Tcr}$ and all spermatids contain the distorters products (regardless of the cell origin).

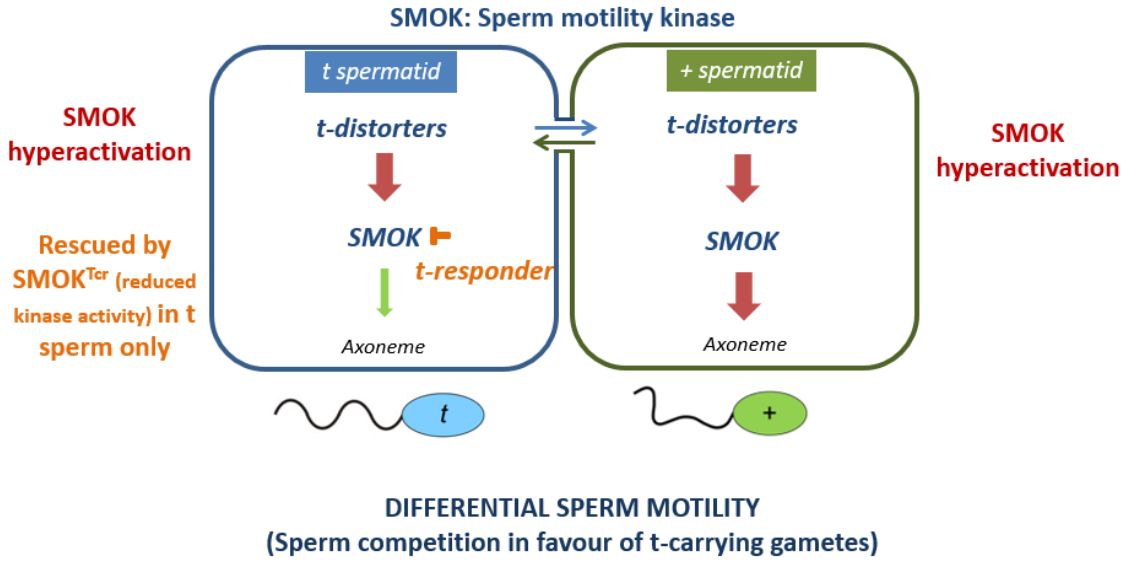

Figure 4: Simplified model of the mechanisms controlling sperm motility and causing TRD. Haploid sperm cells of t-bearing heterozygote are connected by intercellular bridges allowing sharing of the distorter genes products (indicated by arrows). SMOK and $SMOK^{Tcr}$ are not shared between spermatids. The distorter products are causing hyperactivation of SMOK resulting in impairment of flagellar motility in all Smok bearing cells. In t spermatids, which contain $SMOK^{Tcr}$ is hyperactivation suppressed and motility is not harmed. This cascade brings an advantage in favour of t-carrying spermatids.

The distorters are believed to encode components of signalling cascades regulating SMOK, which (as the sperm motility kinase) is suggested to be involved in sperm motility. The products of the distorter genes (TAGAP1, FGD2, NME3) might have an enhancing effect on SMOK activity resulting in abnormal flagellar movement in cells with SMOK. However, the t-haplotype spermatids containing the $SMOK^{Tcr}$ will be spared from abnormal flagellar movement and show normal motility, since $SMOK^{Tcr}$ has the ability to counterbalance the effect of distorter products (Bauer et al., 2012; Herrmann & Bauer, 2012; Herrmann et al., 1999). It causes the superior swimming behaviour of t-haplotype sperm as compared to wt sperm derived from the same sample (Bauer et al., 2012).

1.9. Sperm motility and energetic metabolism

Although a part of the molecular mechanisms responsible for t-haplotype TRD have been already enlightened, complete explanation of this phenomenon is still missing. Differences in sperm motility characteristics between genotypes of t were observed (Olds-Clarke & Peitz, 1985). Interestingly, it was also observed that there are two subpopulations in terms of flagellar motility in sperm form t/+ mice (Amaral, unpublished data).

Identification of sperm motility kinase SMOK variant as the responder suggested that TRD might be caused by alternations is a signalling pathway involved in sperm motility (Herrmann & Bauer, 2012; Herrmann et al., 1999).

Proteome analysis indicates that while in t homozygous samples there is a down-regulation of proteins involved in bioenergetic metabolism (when compared to wild type), sperm from heterozygotes have an up-regulation of several bioenergetic metabolic proteins and down-regulation of others (Amaral, unpublished data).

This so far gathered data led us to current working hypothesis that the differences in motility causing TRD are, among other things on the grounds of metabolic causes.

1.9.1. Adenosine triphosphate (ATP)

Spermatozoa are able to generate energy in form of ATP through aerobic and anaerobic metabolic pathways – Krebs cycle, mitochondrial oxidative phosphorylation and glycolysis (Peña et al., 2009). In the absence of metabolic substrates, sperm consumes its

ATP and subsequently becomes immotile and unable to hyperactivate (Goodson et al., 2012). Hyperactivation is considered to be crucial process for fertilization, since it enhances the abilities of the sperm to detach from the wall of the oviduct, to move and to penetrate the zona pellucida of the oocyte (Suarez & Ho, 2003).

This demonstrates the role of ATP as the energetic storage of the sperm cell. ATP in sperm is used for movement, for fusion events during the acrosome reaction and to transport ions and other molecules through membranes against concentration gradients (Visconti, 2012).

1.9.2. Mitochondrial membrane potential (MMP)

Energy is stored in the mitochondria as a proton concentration gradient and an electric potential gradient across the membrane. These gradients are generated by electron transport maintained by the inner mitochondrial membrane and drive the synthesis of ATP (Kasai et al., 2002). There was found a positive correlation between mitochondrial membrane potential and sperm motility parameters (Troiano et al., 1998). From that reason mitochondrial membrane potential has been used as an indicator of sperm functionality (Amaral & Ramalho-Santos, 2009).

2. Aims

Although some of the molecular mechanisms controlling the postulated sperm competition in favour of t-bearing spermatids have been deciphered, the complete picture of the sperm cellular features operating in TRD is not fully drawn.

The main goal of this project was to determine physiological differences between sperm samples from different genotypes of t.

To achieve this goal, sperm samples from the three genotypes of t (t/t, t/+, +/+) were analysed and compared for ATP levels and Mitochondrial membrane potential.

We also aimed to determine if there is a correlation between sperm cell metabolic characteristics and its variant of t-complex region.

3. Materials and methods

3.1. Biological material

All animals used for experimental work were raised and maintained in Mouse facility of Max Planck institute for Molecular Genetics according to its regulations and guidelines. All used male mice were sexually mature (8 weeks of age as minimum). In each experiment, the different variants of t-complex (t/t, t/+, +/+) were compared. Mice from different genotypes used in each experiment were approximately the same age.

For purposes of wild type (+/+) control, mouse line C57BL/6 was used. As heterozygotes (t/+) and homozygotes (t/t) were used the complete t-haplotypes t^{w5} and t^{w32} in heterozygosity (t^{w5}/+, t^{w32}/+) and homozygosity (t^{w5}/t^{w32}) as congenics on the C57BL/6 genetic background.

3.1.1. Krebs Ringer medium (KR medium)

Krebs ringer medium was used as a basic medium for extraction and manipulation with sperm samples during each experiment.

Table 1: KR medium composition

Reagent	Final concentration
NaCl	118mM
KCl	4.7mM
KH_2PO_4	1.2mM
$MgSO_4$	1.2mM
$CaCl_2$	1.7mM
glucose	5.6mM
Sodium pyruvate	0.3mM
Sodium lactate	21mM
$NaHCO_3$	25mM
Penicillin-streptomycin	100IU/mL, 100µg/mL
BSA	1% (w/v)
HEPES	10mM

All reagents (summed in table 1, purchased from Sigma Aldrich, USA) were added to 40 mL of purified Milli-Q water and mixed until fully dissolved. Subsequently, pH of the solution was measured on pH meter (Thermo Fisher Scientific, USA) and adjusted to reach the range 7.2 – 7.4. Medium was refilled with purified Milli-Q water till 50 mL to reach desired final concentrations and filtered over disposable syringe filter (pore size 0.4 µm, Thermo Fisher scientific, USA) afterwards. Prepared medium was kept at 4°C.

3.1.2. Sperm cell isolation

Male mice were sacrificed by cervical dislocation in general anaesthesia by Isoflurane (Sigma-Aldrich, USA). Testes and epididymides were isolated and purged from fat and connective tissue. Epididymides were separated from testes and transferred to a small Petri dish containing 1.2 mL prewarmed KR medium. Cauda epididymides were isolated and transferred to a new Petri dish containing 1.2 mL prewarmed KR medium. Small nicks were made to the surface of epididymis by a needle. Blood vessels were avoided while penetrating of epidydimal surface to prevent red blood cells contamination. Petri dish with isolated epididymis was incubated at 37°C, 5% CO_2 in Labotect Incubator C16 (Labotect, Germany) for 10 minutes with the purpose of sperm swim-out. After 10 minutes sperm were transferred to an Eppendorf and centrifugated for 1 minute, 100xg at 37°C in Eppendorf Centrifuge 5415R (Eppendorf, Germany). Tissue pieces present in the pellet were discarded.

3.1.3. Sperm concentration determination

10 µL of sperm suspension was loaded into Bright-Line™ haemocytometer chamber (Sigma, Germany). Concentration of spermatozoa was assessed under optical microscope Motic BA310 (Motic, China) at 100X. The number of spermatozoa in random five squares of the haemocytometer was counted three times. The values from three counts were averaged. Resulted number was multiplied by 0.05. The sperm concentration refers to million sperm per 1mL.

3.1.4. Red blood cell (RBC) contamination determination

10 µL of sperm suspension was loaded into Bright-Line™ haemocytometer chamber (Sigma, Germany) and assessed under optical microscope Motic BA310 (Motic, China) at 100X. Red blood cells in the squared field were counted. Resulted number was multiplied by 0.01 which refers to millions of RBC per mL. To calculate the percentage

of contamination, the amount of RBC was divided by the total amount of cells (sum of sperm cell and RBC).

3.2. ATP levels

3.2.1. 3.2.1. ATP extraction

To determine the best procedure of ATP extraction from sperm sample for this experiment, various alternatives of sample concentration and extracting medium were tested. Efficiency of extraction was observed in between sample diluted in water, Tris-EDTA buffer and Reaction buffer. Not diluted sample and sample diluted by these buffers in ratio 1:5 and 1:10 were compared. Three different tested sample concentrations in three different solutions resulted in 9 samples which were compared in matter of ATP extraction efficiency. Optimized protocol is described below.

An Eppendorf containing 450 µL of Tris-EDTA buffer was prepared for each sample. Tubes with Tris-EDTA buffer were placed in Eppendorf Thermomixer Comfort (Eppendorf, Germany) set at 100°C until the Tris-EDTA buffer was boiling. Subsequently, purified sperm sample was diluted 1:10 by diluting 50 µL sperm sample in 450 µL of preheated Tris-EDTA buffer. Samples in Tris-EDTA buffer were boiled in Eppendorf Thermomixer for another 5 minutes. After this period, sperm samples were pelleted by centrifugation at 16 000 xg for 5 minutes in Eppendorf centrifuge 5415R (Eppendorf, Germany). The supernatant was transferred into new Eppendorf tubes. Extracted ATP samples were placed on ice immediately.

3.2.2. 3.2.2. ATP levels measuring

To measure ATP concentration in each sample, ATP Determination Kit (Molecular Probes, USA) was used. This commercial kit provides a recombinant firefly luciferase, as an oxidative enzyme that produces bioluminescence, and its substrate D-luciferin. The assay is based on luciferase´s requirement for ATP in producing light (*Figure 5: ATP*

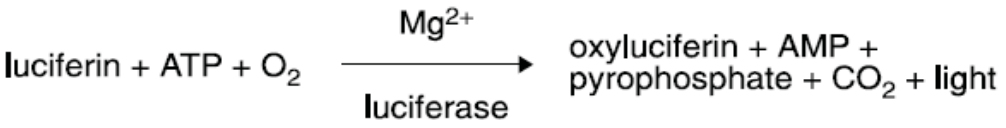

Figure 5: ATP Determination Kit - principle

Determination Kit - principle). Reaction solution was prepared according to manufacturer´s indications (described in table 2).

Table 2: ATP Determination Kit, reaction solution

Reagent	**In 10 mL Reaction solution**
D-luciferin 10mM	0.5 mL (final conc. 0.5 mM)
Luciferase, firefly recombinant	2.5 µL
Dithiothretiol (DTT) 0.1M	0.1 mL (final conc. 1mM)
20x Reaction buffer	0.5 mL (final 1xRB)
dH_2O	8.9 mL

Each ATP sample was added to this reaction solution. Resulting bioluminescence was measured using 96-well GloMax -Multi Detection System luminometer (Promega, USA). Measuring for each sample was made in technical triplicates and resulting values were averaged. As a negative control, technical triplicate with the reaction solution and sample replaced by dH_2O was used. Luminescence signal from negative control was subtracted from results as a blanc. The ATP content of each sample was calculated from a series of ATP standards run in triplicates which was present by every single measuring. ATP content standard curve was composed of 7 standards with the first positive standard of 25 nM ATP concentration and the last standard of 0.4 µM ATP content (described in Table 3: ATP standards).

Table 3: ATP standards

Standard	ATP	Concentration of ATP
0	0 pmol	0
1	0.25 pmol /10 µL	25 nM
2	0.5 pmol / 10 µL	50 nM
3	1 pmol / 10 µL	0.1 µM
4	2 pmol / 10 µL	0.2 µM
5	3 pmol / 10 µL	0.3 µM
6	4 pmol / 10 µL	0.4 µM

Only those measuring were accepted, where R-square of the standard curve reached above 0.95 (R^2>0.95).

Sperm concentration for each sample was determined (described in 1.3.) Red blood cell contamination of all samples was determined (described in 1.4.). The ATP content of each sample was calculated based on known number of cells entering the ATP extraction. Results were expressed in pmol ATP/10^6 spermatozoa.

3.3. Quantitative analysis of mitochondrial membrane potential

3.3.1. 3.3.1. JC-1 staining

In order to evaluate mitochondrial membrane potential in samples of sperm cells from tested genotypes of t complex, MitoProbe JC-1 Assay Kit for Flow Cytometry (Molecular probes, USA) was used. Samples stained with this commercial kit were subsequently analysed using Flow Cytometry (BD FACSAria™ II, BD Biosciences, USA).

The main component of MitoProbe JC-1 Assay Kit is the 5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolyl-carbocyanineiodide (JC-1). This lipophilic cationic fluorescent carbocyanine dye possess the ability to form multimers known as J-aggregates after accumulation in mitochondria with high membrane potential. In mitochondria with low membrane potential JC-1 remains in monomeric form. The fluorescence spectra of JC-1 in the monomer form show an excitation and emission peak in the green range (510-530 nm). The emission of J-aggregates is in the high orange-red range wavelength (590 nm). This fluorescence emission shift from green to red enables us to distinguish the spermatozoa with low and high mitochondrial membrane potential. Moreover, the red/green fluorescence intensity ratio provides an information about the level of mitochondrial depolarization.

The kit also includes the mitochondrial membrane potential disrupter, CCCP (carbonyl cyanide 3-chlorphenylhydrazone). Together with JC-1 fluorescent dye, the cells exposed to both reagents serve as a control to confirm that the JC-1 response is sensitive to changes in membrane potential.

Before sperm samples were processed, JC-1 dye solution was prepared by dissolving in DMSO. Every sample was processed with positive and negative control. Since JC-1 dye was dissolved in DMSO, an equal volume of DMSO was included in the unstained controls.

Concentration of extracted sperm samples was determined (described in 1.3) and volume of sample answering to 1 million of sperm cells was added to prewarmed KR medium to reach final volume 1 mL. CCCP was added to positive control (50 µM final concentration). Sample and controls were incubated at 37°C, 5% CO2 in Labotect Incubator C16 (Labotect, Germany). After incubation period, JC-1 was added to the sample and controls in 2 µM final concentration. To maintain the most possible similar conditions, DMSO of the same volume was added to negative control. The sample and the control were incubated for 20 minutes in 37°C, 5% CO2. Afterwards, both the sample and the controls were centrifuged for 5 minutes, 0.4 rcf (400 xg) in Eppendorf centrifuge 5415R (Eppendorf, Germany). Supernatant was discarded and substituted with KR medium with the purpose of cell resuspendation. Access of light was avoided during the whole procedure.

3.3.2. Flow cytometry analysis

Fluorescent assessment of sperm mitochondrial membrane potential using JC-1 has been quantified by flow cytometry. All processed samples were analysed using BD FACSAria™ II Analyser (BD Biosciences, USA). Data from Flow cytometer were processed by software DIVA 6.3.1.

Prior to flow cytometry, stained samples were filtered through a 50mm nylon mesh filter. In the flow-cytometer, the fluorescence signal was exploited to count and sort individual cells bearing the fluorescence marker. Used configuration was 488 nm laser (GFP detection) with pre-filter 505LP and filter 510/20 together with 561 nm laser (mCherry detection), pre-filter 600LP, filter 61/20-25.

3.4. qPCR analysis of JC-1 sorted samples

3.4.1. 3.4.1. Flow cytometry sorting

Sperm sample was processed by MitoProbe JC-1 Assay Kit for Flow Cytometry (described in 1.6) and analysed by flow cytometry (described in 1.6.2).

Red fluorescence of JC-1 exposed sample was analysed and subsequently a subpopulation on the top of fluorescence intensity scale was singled out. Secondly, a subpopulation of lower red intensity was singled out. Sorting was performed till

100 000 sorted events in each sorted group. As a control, cell subpopulation of the same size 100 000 cells was sorted without any fluorescence restrictions. The internal diameter for the nozzle was 85 μm.

After sorting, the samples were centrifuged in Eppendorf centrifuge 5415R (Eppendorf, Germany) and the supernatant was replaced by KR medium. Subsequently, DNA was extracted from sorted subpopulations.

3.4.2. DNA extraction

To gain the optimal method of DNA extraction with the best proceeds of DNA extracts, different methods of DNA extraction were compared. Following protocol was concluded as best results providing.

QIAamp® DNA Micro Kit (QIAGEN, Germany) was used for DNA extraction from sorted subpopulations of spermatozoa and from mice tails according to suggested protocols. Concentration of resulting DNA samples was measured by Qubit™ dsDNA HS assay Kit (ThermoFisher Scientific, USA) on NanoDrop 3300 Fluorospectrometer (Thermo Scientific™, USA).

1. Sample in 1.5 mL Eppendorf tube was centrifuged for 5 minutes, 2000 xg
2. Supernatant was discarded
3. 300 μL buffer AE was added
4. Eppendorf tube with the sample was subsequently centrifuged for 5 minutes, 2000 xg
5. Supernatant was discarded
6. 280 μL buffer AL, 10 μL proteinase K and 10 μL 1M DTT were added
7. Eppendorf tube was mixed by pull-vortexing for 10 second
8. Sample was placed at 56°C, 900 rpm shaking for at least 1.5 hour in case sperm cells, overnight in case of somatic cell samples
9. After short centrifugation, 300 μL buffer AL was added
10. Eppendorf tube was mixed by vortex for 10 second
11. Sample was incubated for 10 minutes (70°C, 900 rpm) and vortexed every 3 minutes
12. Eppendorf tube was centrifugated (16000 xg) for 1 minute
13. Supernatant was transferred into new 1.5 mL Eppendorf tube

14. 150 µL 96-100% ethanol was added
15. Eppendorf tube was mixed by vortex for 15 second and briefly centrifuged
16. Content of the Eppendorf tube was transferred to QIAamp MiniElute column
17. Column was centrifuged (6000 xg) for 1 minute
18. Column was placed in a clean 2 ml collection tube and collection tube containing the flow-through was discarded
19. 500 µL buffer AW1 was added
20. Column was centrifuged (6000 xg) for 1 minute
21. Column was placed in a clean 2 ml collection tube and collection tube containing the flow-through was discarded
22. 700 µL buffer AW2 was added
23. Column was centrifuged (6000 xg) for 1 minute
24. Column was placed in a clean 2 mL collection tube and collection tube containing the flow-through was discarded
25. 700 µL 96-100% ethanol was added
26. Column was centrifuged (6000 xg) for 1 minute
27. Column was placed in a clean 2 mL collection tube and collection tube containing the flow-through was discarded
28. Column was centrifuged for 3 minutes (16000 xg)
29. Collection tube was replaced by regular 1.5 mL Eppendorf tube
30. Column was incubated at room temperature with an open lid for 20 minutes
31. 50 µL AE buffer was applied to the centre of column´s membrane
32. Column was incubated at room temperature for 1 minute
33. Column was centrifuged for 1 minute (16000 xg)
34. Resulting DNA sample was collected in 1.5 mL Eppendorf tube and column was discarded

3.4.3. qPCR optimization

3.4.3.1. primer design

In order to distinguish DNA extracted from sperm cell carrying t-haplotype and from sperm cell carrying wild type form of the region, two qPCR essays were established. During primer design, we took advantage of the deletion located in the position 17:6753937-6753971 in the sequence of t haplotype, spanning 34 base pairs, which is not

present in wild type variant of the region (Reynolds & Planchard, 2004). The specificity of the assays is supposed to be handled by the primers hb434-2 and hb435-2.

Primer hb-434-2 is designed as exclusively binding to t-haplotype DNA region. It is spanning over both edges of the deletion in t haplotype sequence and therefore it is complementary only with this variant. It is coupled with primer hb-459vil-s and provides t-specific assay.

Primer hb-435-2 is supposed to be exclusively binding to wt version of DNA sequence. As such, it is partly overlapping the sequence, which is present only in wild type variant and is deleted in t-haplotype sequence. Together with hb460vil-as provides wild type (wt) specific assay.

Primers for t- haplotype targeted qPCR assay:

hb459vil-s	5′-TCATGGACCAACACAAGCTC-3′
hb434-2	5 -ACCTGACTGGAAATGGCAGG-3′

Primers for wt targeted qPCR assay:

hb460vil-as	5′CACAAAACTGAAATCTCCCTCTC-3′
hb435-2	5 -GTGCCTGCCTTTTCAGTGTG-3′

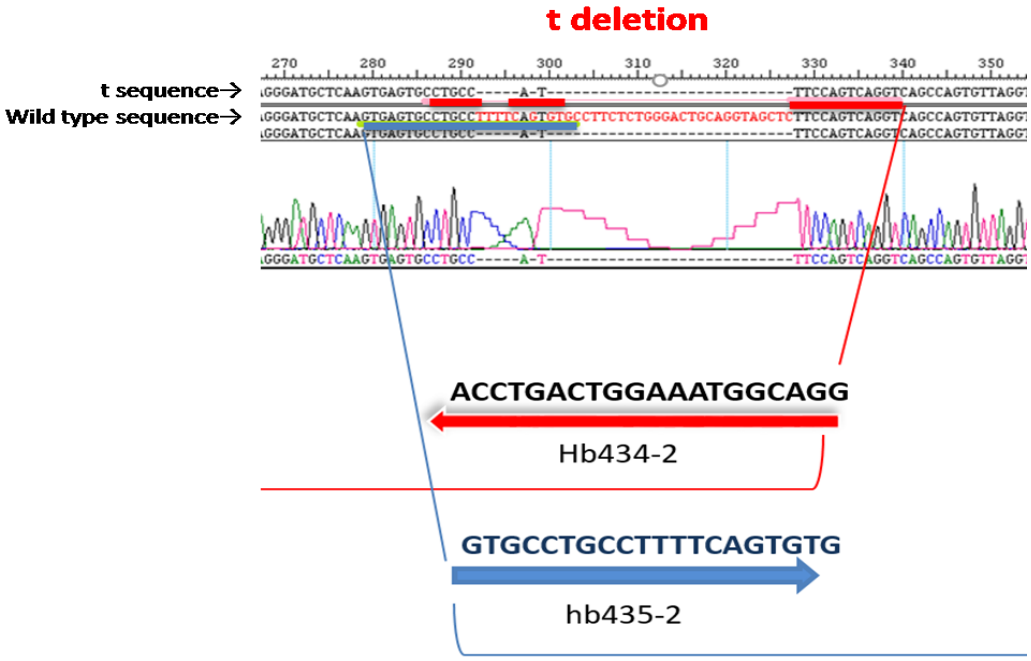

Figure 6: Primer design, designed by Hermann Bauer; region deleted in t allele and present in wild type allele labelled red; Hb434-2 and hb435-2 primers shown as allele specific, hb434-2 (red arrow) amplifying t-haplotype sequence exclusively, hb435-2 (blue arrow) targeted on wild type sequence only

Specificity of designed primers was repeatedly tested on DNA extracted from tails. Subsequently, primers were tested on DNA extracted from sperm.

3.4.3.2. "Tail proof" and "Sperm proof" assay testing

To test the specificity of the assays, repeated testing was performed with both DNA sources. The test included standard curve made of DNA extracted form heterozygous t/+ sample analysed in different dilutions. This series of different DNA concentration analysed by two different qPCR assay at the same time provided two standard curves. The efficiency of the resulting standard curves was compared.

The test as well contained DNA samples extracted from t/t homozygote and +/+ homozygote. DNA sample of t/t homozygote served as a positive control for t assay and negative control for wt assay. DNA sample of +/+ homozygote served as a positive control for wt assay and negative control for t targeted assay.

Next point of the test were DNA samples from +/+ and t/t homozygotes mixed in known ratios (1:3, 3:1, 1:1). DNA concentration of used samples was measured by Qubit™ dsDNA HS assay Kit (ThermoFisher Scientific, USA) on NanoDrop 3300 Fluorospectrometer (Thermo Scientific^{TM}, USA), preciously diluted and mixed into mixtures of desired ratios. As a result, the same ratio of Ct values was expected.

Each test contained as well negative control with all reagents except DNA in the same final volume (NTC).

3.4.3.3. qPCR optimization process

In both types of DNA sources, different concentrations of DNA inserted for qPCR were tested by gradual dilution of the same DNA sample.

Primer concentration was tested by dilution cascade of primer couple solution. 5 µM, 2.5 µM, 1.25 µM and 0.75 µM final concentrations of each primer in the reaction solution were tested on the same DNA source, once DNA extracted from tail, once DNA extracted from sperm. 1.25µM was concluded as the final smallest possible and not inhibiting primer concentration.

Because of persisting difficulties, different methods of DNA extraction were tested and compared in the matter of qPCR assay results. As well, qPCR performed on DNA extracted from sorted and unsorted cells by flow cytometry was compared.

3.4.4. qPCR analysis of sorted samples

All qPCR runs were performed on StepOne Plus RealTime PCR system (ThermoFisher Scientific, USA). Each run contained two assays targeted on different regions.

All performed qPCR assays were composed of GoTaq® qPCR Master mix (Promega, USA) complemented by CXR reference dye, and primer couples in 1.25µM final concentration (Eurofins, Germany) (Table 4: qPCR reaction solution).

Table 4: qPCR reaction solution

Component	Volume (µL)
GoTaq® qPCR Master mix	10
Primer mix	5
DNA template + nuclease free H_2O	5
Total volume	**20**

Table 5: qPCR setup

1.1.Temperature	Duration
95°C	2 min
95°C	15s (40x)
60°C	30s (40x)
95°C	15s
60°C	15s

The Samples were tested in technical triplicates, final Ct value was averaged. Negative control (NTC) containing all reagents except targeted DNA was always present to both assays. Tested samples were subjected to melting curve analysis.

The relative quantification of the Ct values was calculated by using StepOnePlus™ Software 2.3 (ThermoFisher Scientific, USA).

During final qPCR analysis of DNA form sorted samples, control assay targeted to Mesogenin (Msgn1) region (Eurofins Genomic, Germany) was added to the t region targeted assay. Wt targeted assay was excluded. Msgn1 assay is a good result providing,

well established assay targeted on protein coding region on mouse chromosome 12. It provides information about total DNA amount in the sample. During this experiment, Msgn1 assay was used as the control tool reflective of real amount of amplified DNA in the sample. Differences of outcoming fluorescence signal caused by deviation from expected DNA concentration were adjusted to the fluorescence signal of Msgn1 control.

With amount of DNA verified by Msgn1 qPCR assay, it was possible to adjust fluorescence signal results from t targeted qPCR assay and as interpret them more clearly.

DNA extracted from sorted cell subpopulations (group of metabolically more active cells and contrarily group of metabolically less active cells) was assessed by qPCR assay targeted on t allele and compared to DNA extracted from the same amount (100 000) of not sorted cells. These DNA samples were as well processed by Msgn1 targeted assay for control purposes.

3.5. Gel electrophoresis

Amplified samples were run on 4% agarose gel with the purpose of identifying the length of amplified fragments. Each gel was prepared by heating 1.5 g low melting NuSieve® GTO® Agarose powder (Lonza, Czech Republic) and 2.5 g Biozym LE agarose powder (Biozym, Germany) in 100 mL 1xTBE buffer in a microwave. After cooling, 5 µL of SYBR® Green I Nucleic Acid Gel Stain (Invitrogen, USA) was added. Solution was poured into a mould and left at room temperature until solid. 15 µL of PCR product was mixed with 2 µL of 10x DNA Gel loading dye (thermo Fisher Scientific, USA). Resulting mixture of PCR product and loading buffer was loaded onto the solid agarose gel. 10 µL of 1Kb Plus DNA Ladder (Invitrogen, USA) were run simultaneously with the samples to distinguish their molecular weight. Process of electrophoresis was run at 90 V for 35 minutes. Subsequently, each gel was photographed by Gel Doc™ XR+ Gel Documentation System (Bio Rad, Germany).

3.6. Statistical analyses

Statistical analyses were performed using GraphPad Prism Version 5.0 (GraphPad Software, Inc., CA, USA). The different genotypes were compared for both ATP levels and JC-1 outcomes using Kruskal-Wallis test followed by Dunn´s multiple comparison test. P values < 0.05 were considered significant.

4. Results

4.1. ATP levels

We succeed in establishing stable method of ATP level assessment from sperm samples. After a period of ATP extraction method optimization and measurement technique calibration we achieved stable outcomes, which were possible to observe during each measuring via standard curve of ATP levels.

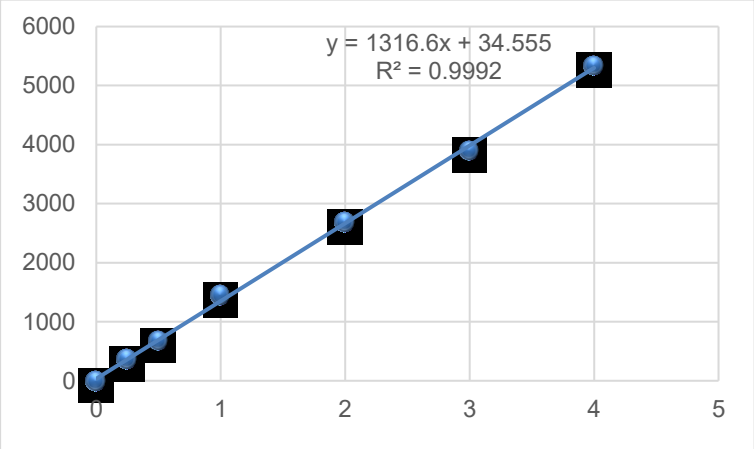

Figure 7: ATP standard curve; x axis ATP (pmol), Y axis luminescence; R^2=0.9992.

Within this experiment, ATP contents of 10 sperm samples from each homozygote t/t, heterozygote t/+ and wild type +/+ (n=30 sperm samples in total) were measured by luminometer.

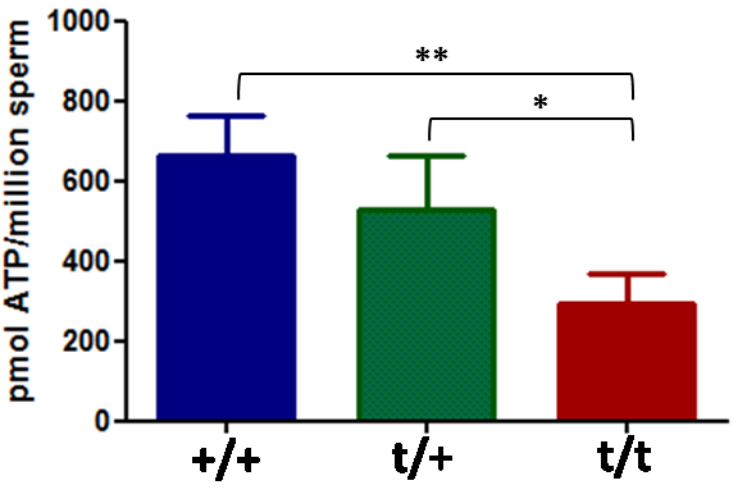

*Figure 8: Sperm ATP levels from +/+, t/+ and t/t mice. Bars represent means + SEMs of pmols of ATP per million of sperm (n= 10 mice for each genotype). Asterisks denote statistical significant differences between genotypes (**P < 0.01; *P < 0.05).*

Significant difference in pmol ATP per million sperm was found between homozygotes t/t and heterozygotes t/+ (p=0.0288) as well as between homozygotes t/t and wild type +/+ samples (p=0.0021). These numbers are leading us to the information, that homozygous sperm samples contain significantly less ATP than samples from other two tested groups. No significant difference was revealed between heterozygotes t/+ and wild type +/+ samples.

4.2. Quantitative analysis of mitochondrial membrane potential

Flow cytometry analysis provided well defined population of single cell measured events by discrimination of other cell types and double events.

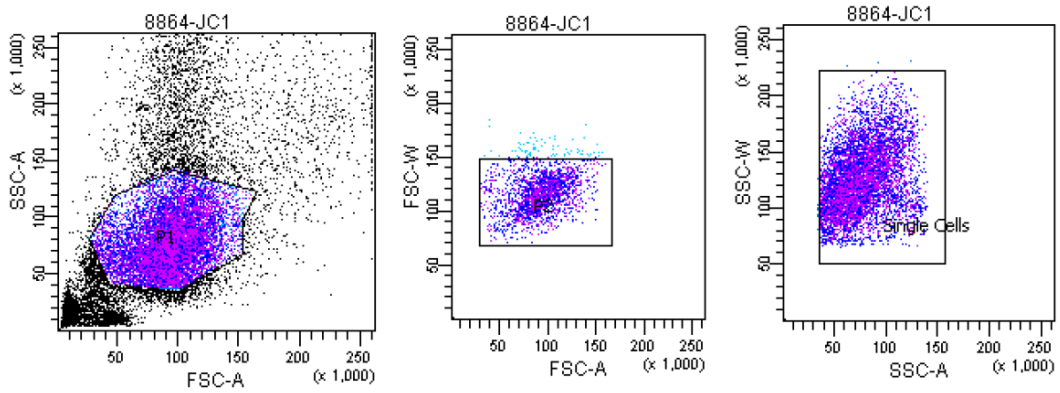

Figure 9: Dot plot JC-1 stained sperm sample, target group definition.

Correct handling of the samples with the commercial kit was proved by coveted result of included controls.

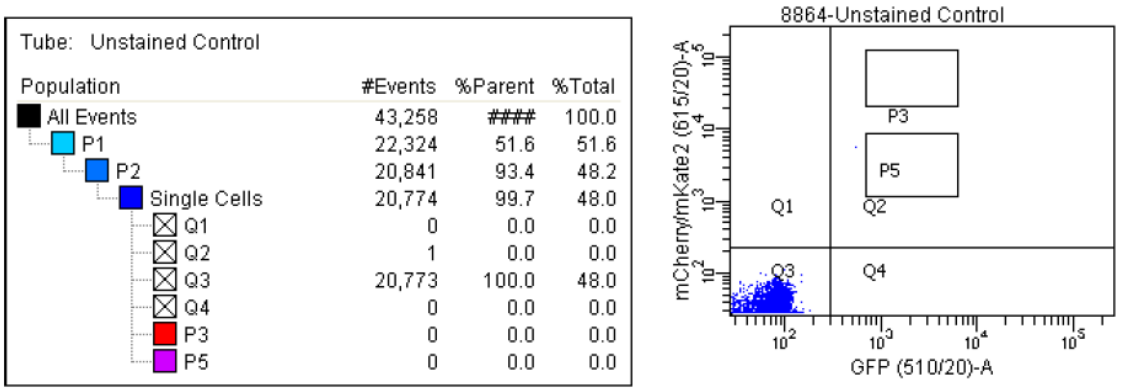

*Figure 10: Flow cytometry analysis, **unstained control example**, dot plot of sperm population GFP vs mCherry (green vs red fluorescence intensity) characteristic.*

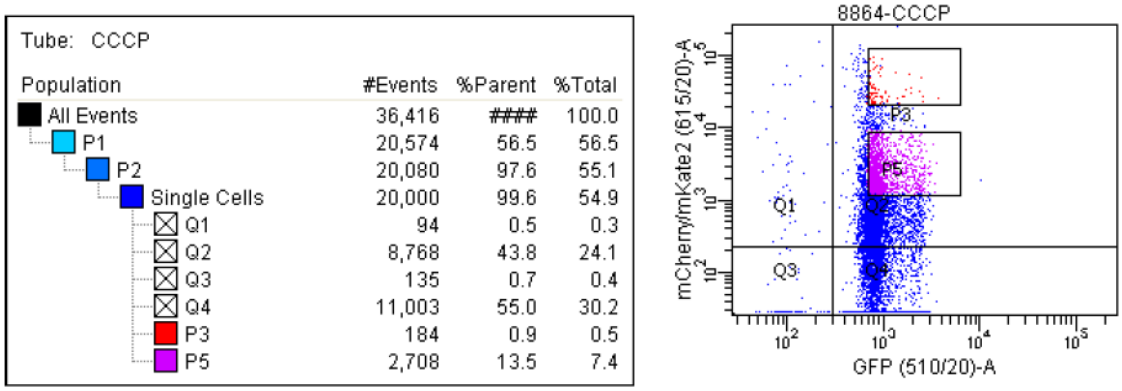

Figure 11: Flow cytometry analysis of JC-1 stained sample, dot plot of sperm population GFP vs mCherry (green vs red fluorescence, ***sample incubated with CCCP*** *mitochondrial membrane disruptor.*

Mitochondrial membrane potential of 10 sperm samples from each genotype of t (n=30 samples in total) was assessed. Flow cytometry has provided information about red to green intensity, which was compared between t genotypes afterwards.

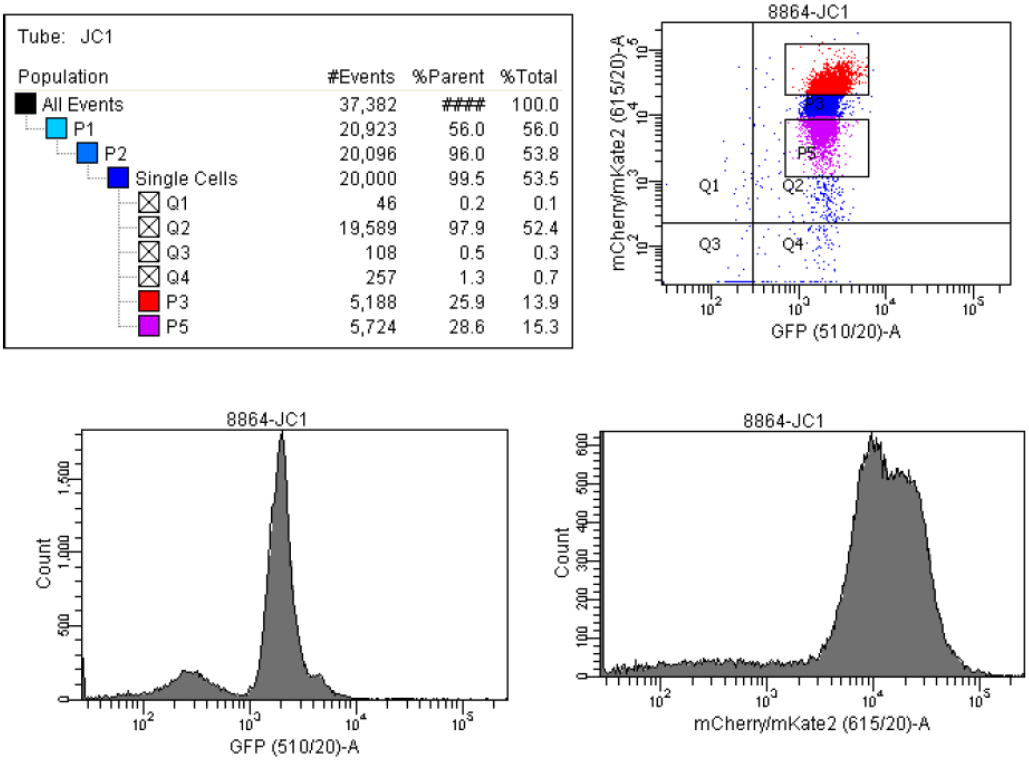

Figure 12: ***Flow cytometry analysis of JC-1 stained example****, histograms of red (mCherry) and green (GFP) fluorescence intensity for stained sperm cells; Dot plot display of the sperm population according to GFP vs mCherry characteristics.*

ANOVA test comparison of red to green intensity values between different t genotype groups of samples provided us following results. Significant difference in red to green intensity ratio was found between homozygote t/t samples and wild type +/+ samples (p=0.0049). Even more noticeable difference (p=0.0080) was observed in comparison of homozygous t/t samples and heterozygous t/+ samples. No significant difference was observed between heterozygous t/+ and wild type +/+ samples. These results are enlightening lower values of MMP in sperm cells of homozygotes t/t.

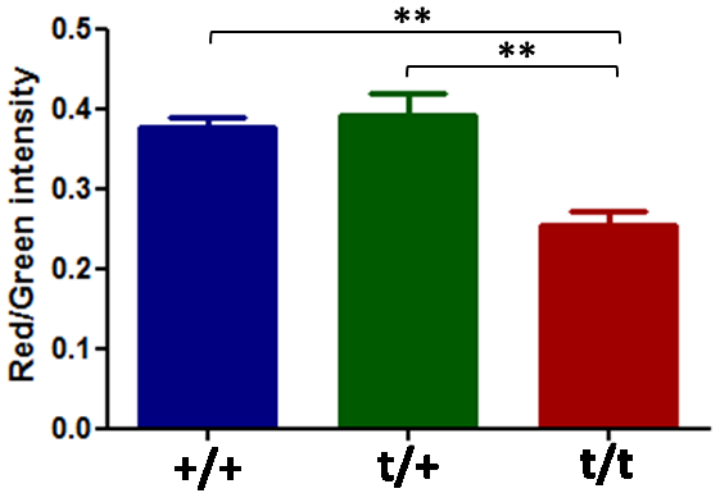

Figure 13: ***Mitochondrial membrane potential analysis*** *in sperm from +/+, t/+ and t/t mice. Bars represent means + SEMs of the ratios of JC-1 red-to-green intensities (n= 10 mice for each genotype). Asterisks denote statistical significant differences between genotypes (**P < 0.01).*

4.3. qPCR analysis of JC-1 sorted samples

During this experiment, we aimed to find out, if there is a correlation between sperm cell´s metabolic characteristics and it´s genotype of t allele. While working on it, we managed to establish useful tool for analysis of t-haplotype DNA samples (**Error! Reference source not found.**

During optimization process, we succeed in providing two qPCR assays analysed in the same plate next to each other with efficiencies similar enough to compare fluorescence signal (Ct values) in between these assays. At the beginning of the testing process, melting curves of both assays were checked and considered correct, but not recorded.

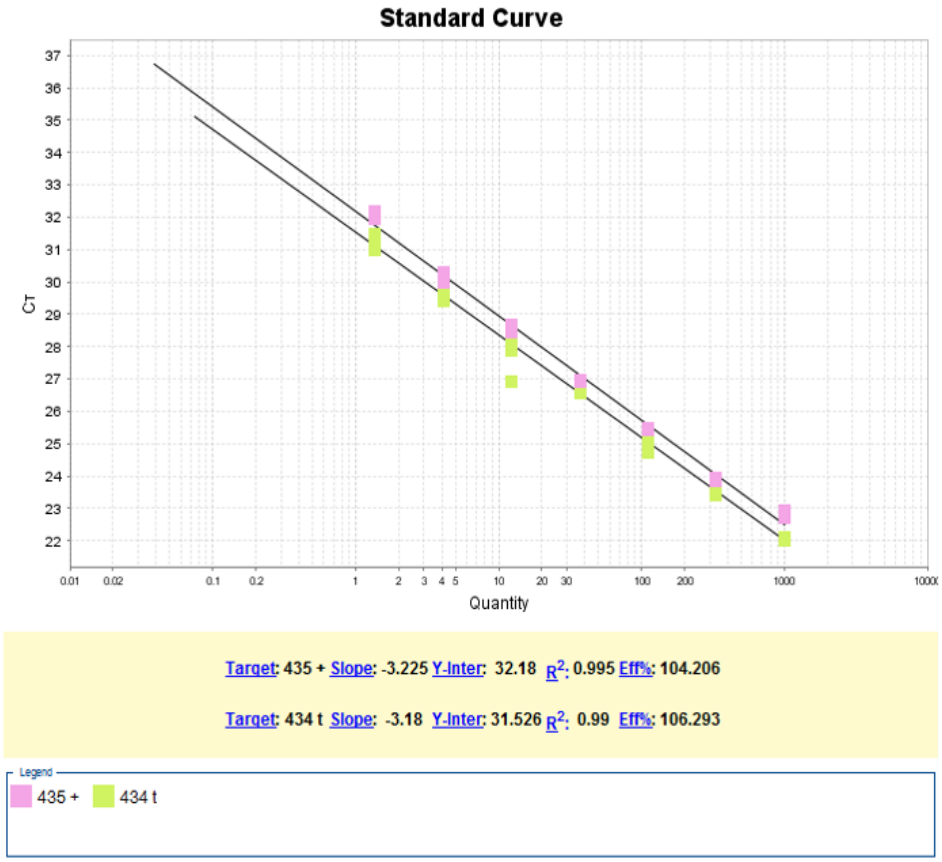

Figure 14: ***Standard curve*** *made of 7 different concentrations of DNA extracted from heterozygous t/+ sample; two qPCR assays; Pink labelled = wt targeted assay, efficiency 104.2%; Green labelled = t targeted assay; efficiency 106.3%.*

We also proved, that it is possible to use even single assay to provide information about DNA sample of unknown ratio of t and wt variant. By using t targeted assay only on 5 artificially prepared samples, it is possible to generate a basic two-point standard curve. This standard curve contains DNA from t/t homozygote representing 100% of possible DNA contained in the sample (of precisely prepared known concentration) and DNA from +/+ wild type DNA sample which represents 0% of possible DNA content of the sample – aka possible contamination. On generated curve are shown resulting amounts of DNA from three artificially prepared mixtures in correct row according their content of t haplotype variant. Resolution of this method does not enable us to conclude concrete ratios, but it provides relative comparison of the samples in between each other. Also, by knowing the Ct value of 100% t haplotype DNA in the control sample, we can generate

approximate percentage of the t haplotype in tested sample relatively to control (**Error! Reference source not found.**).

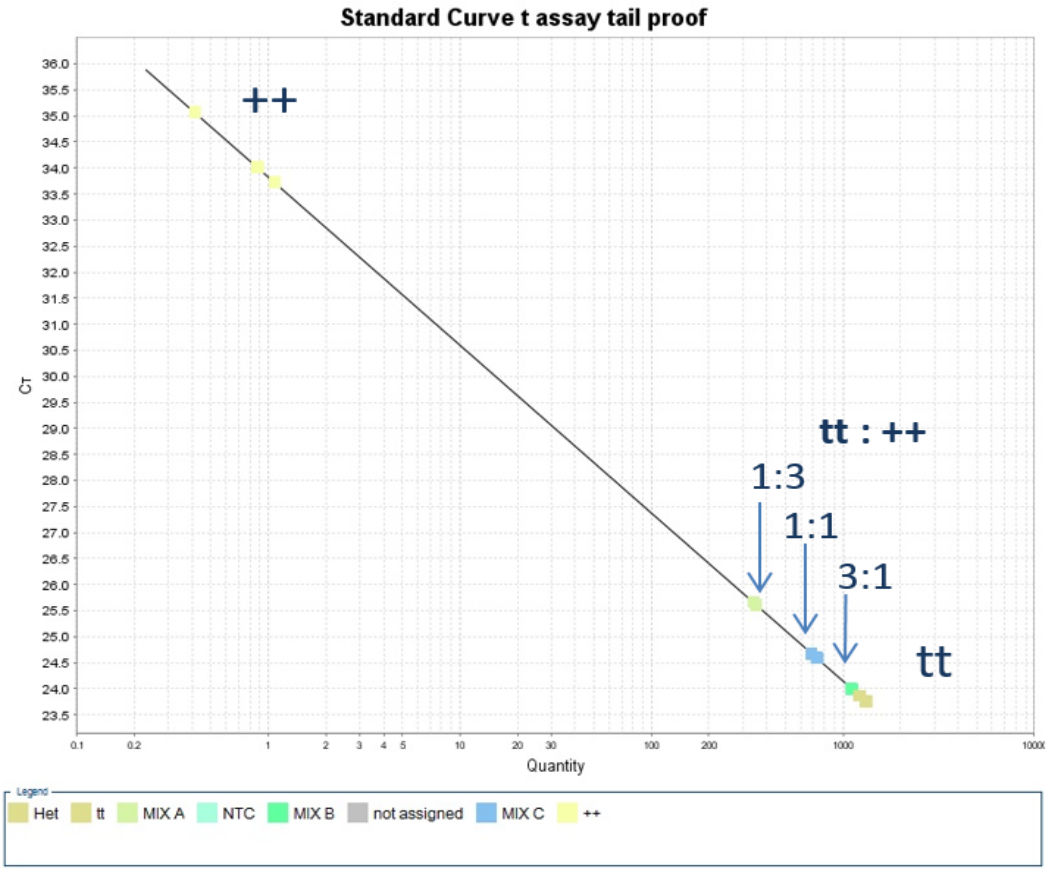

Figure 15: ***Standard curve of qPCR assay targeted on t haplotype****; Minimum Ct (maximum signal) represented by t/t sample, maximum Ct (minimum signal) represented by +/+ sample; artificial mixtures in between on the line.*

Mix	Mix t/t : +/+	expected % of signal compared to signal from t/t	Result %
Mix A	1:3	25%	33%
Mix B	3:1	75%	94%
Mix C	1:1	50%	60%

Figure 14: ***t targeted assay test in percentages;*** *artificially prepared mixtures of DNA extracted from tt and ++ homozygotes; expected percentages of maximal fluorescence; obtained percentages of maximal fluorescence.*

Since the wt targeted assay provided inconsistent result, a series of optimizing experiments was performed. Based on performed primer concentration test, concentration of primers was lowered to 1.25 μM. Comparison of diverse sources of DNA (sperm DNA versus DNA extracted from tail) was performed with no difference found. As well, different concentration of DNA entering the assays were compared. It was proved that lower DNA amount in the qPCR improves the quality of the assay. Different methods of DNA extraction were used and compared on DNA extracted both from tail and sperm. These methods of DNA extraction were compared in matter of qPCR results with no difference observed. DNA extracted from sperm cells that underwent sorting process in flow cytometry device was compared with DNA extracted from not sorted samples in matter of qPCR results. There was found no difference between these samples.

During optimization process, contamination from unknown sources repeatedly appeared and disappeared. Therefore, melting curves of both tested assays were controlled again. Based on melting curves and electrophoresis gels, contamination in wild type assay was concluded too high and exclusivity of the assay was doubted.

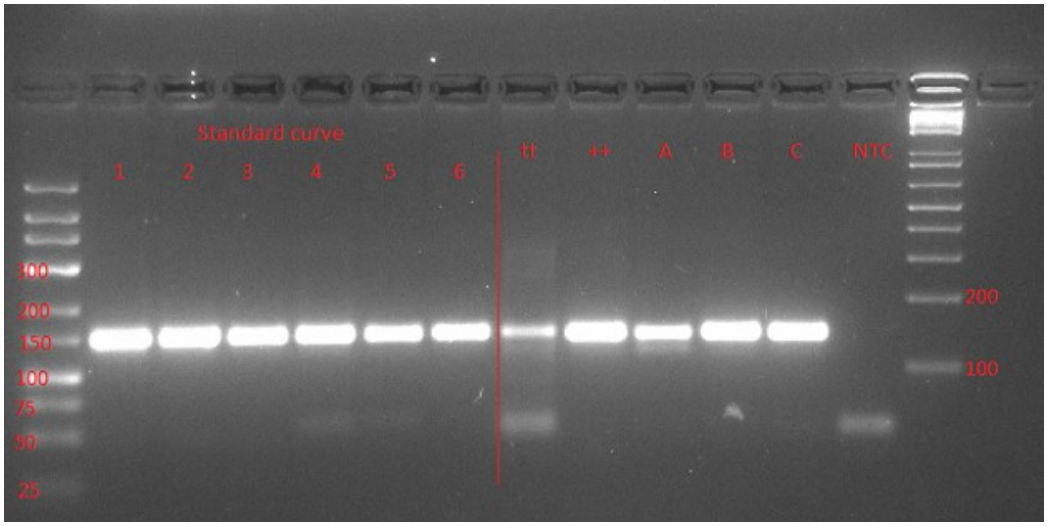

*Figure 17: Electrophoresis gel (4%) of **wild type targeted qPCR assay** showing standard curve made of heterozygous sample t/+ in columns 1-6, **DNA from t/t homozygous sample in tt marked column with visible amplicon in two clear bands**, DNA from +/+ wild type sample as positive control in column labelled ++, artificially prepared mixtures from t/t and +/+ DNA samples in columns labelled A, B and C; non-template control with visible band of contamination amplicon in column labelled NTC; two different ladders of bp marked by numbers on both sides.*

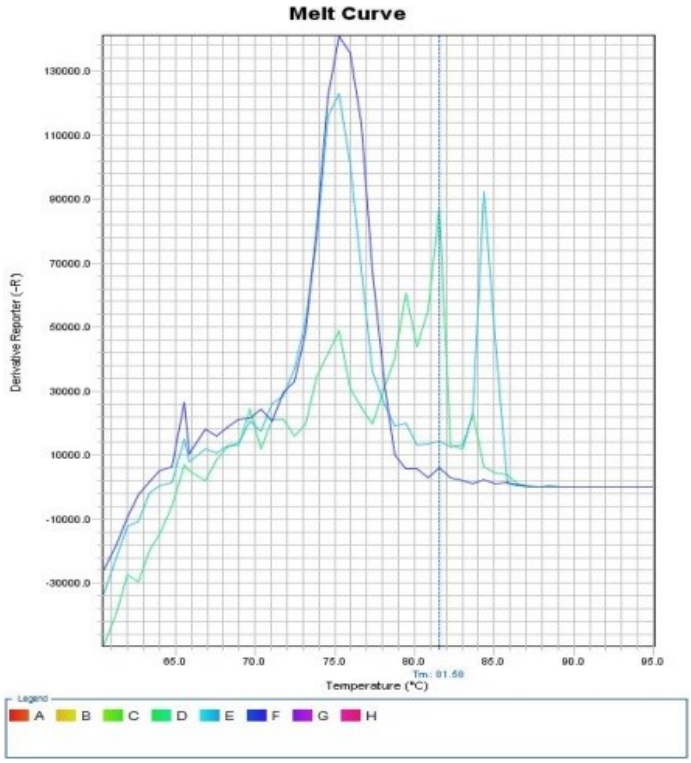

Figure 18: ***Melt curve of t/t sample assessed by wild type targeted assay****; obvious contamination where no amplicon should be observed, main amplicon marked at 81.58°C.*

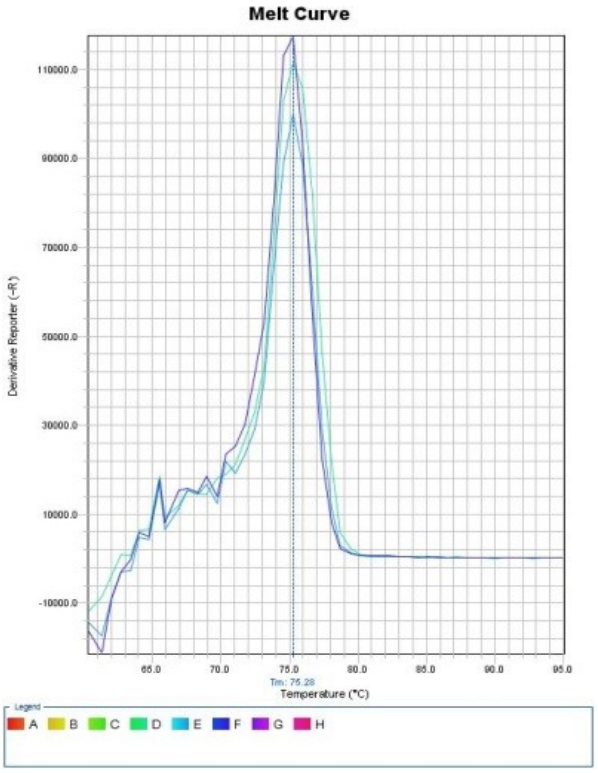

Figure 15: ***Melt curve of non-template control in wild type targeted*** *assay showing contamination with main amplicon marked at 75.28°C.*

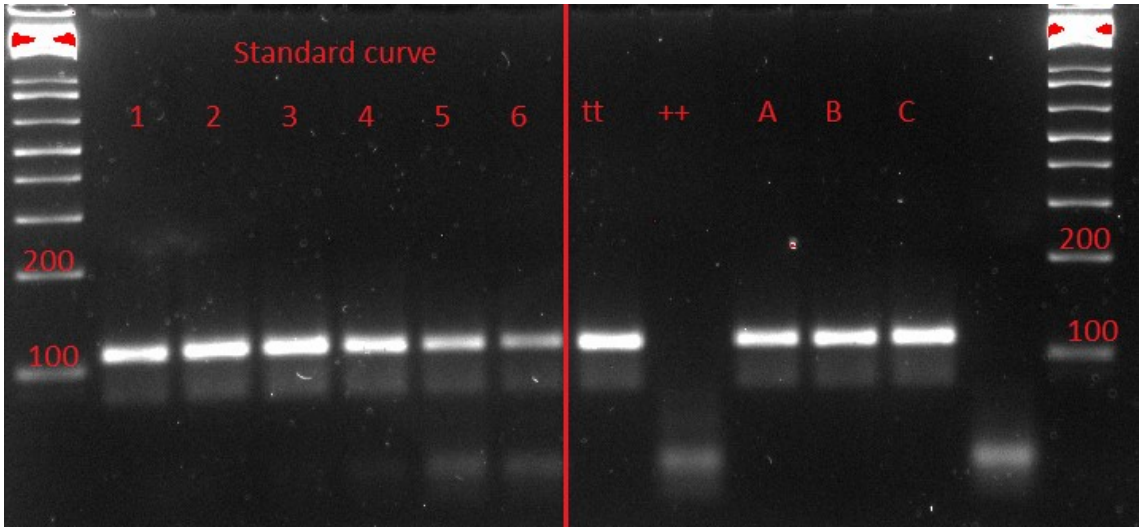

Figure 20: Electrophoresis gel (4%) of ***t haplotype targeted qPCR assay*** *showing standard curve made of heterozygous sample t/+ in columns 1-6, t/t homozygous DNA amplicon in tt marked column as a positive control,* ***DNA of wild type homozygous sample in ++ marked column with amplicon on the same level as in non-template control*** *(last column)****,*** *a series of artificially prepared mixtures of t/t and +/+ DNA (1:3, 3:1, 1:1) in columns labelled A, B, C; non template control in last unlabelled column.*

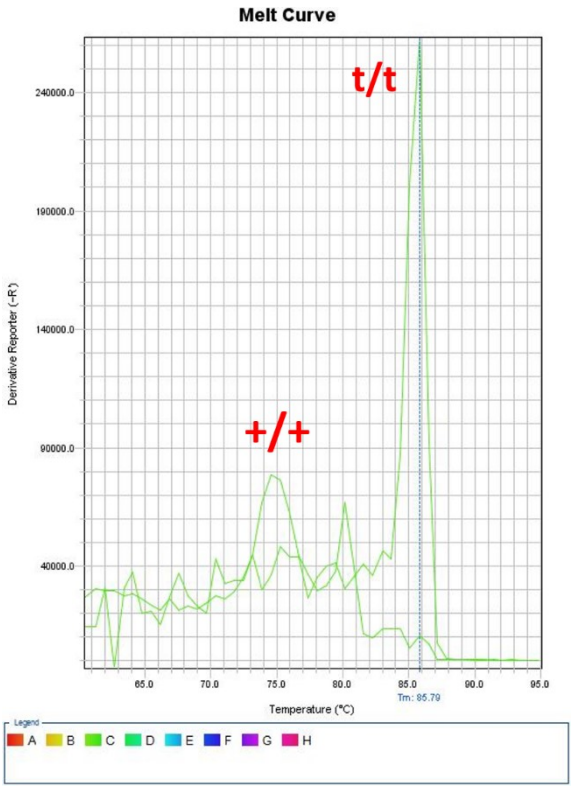

Figure 21: ***Comparison of melt curves of t/t and +/+ homozygous samples*** *(****Error! Reference source not found.****) from t haplotype targeted assay; t/t sample serves as a positive control showing the main amplicon level on 85°C; +/+ sample represents negative control, which has an amplicon on the level of contamination from non-template control (75°C).*

Because of persisting difficulted during optimization process caused by contamination from unknown sources, we were forced to rely on single assay only. This t haplotype targeted assay was supported by adding Mesogenin control assay which allowed even more precise reflection of total DNA amount contained in the ongoing assay.

With the gained certainty about the amount of DNA in the sample, it is possible to conclude relative amount of t variant in the sample by comparing tested sample to the heterozygous t/t not sorted control, which represents 50% of total amount of DNA.

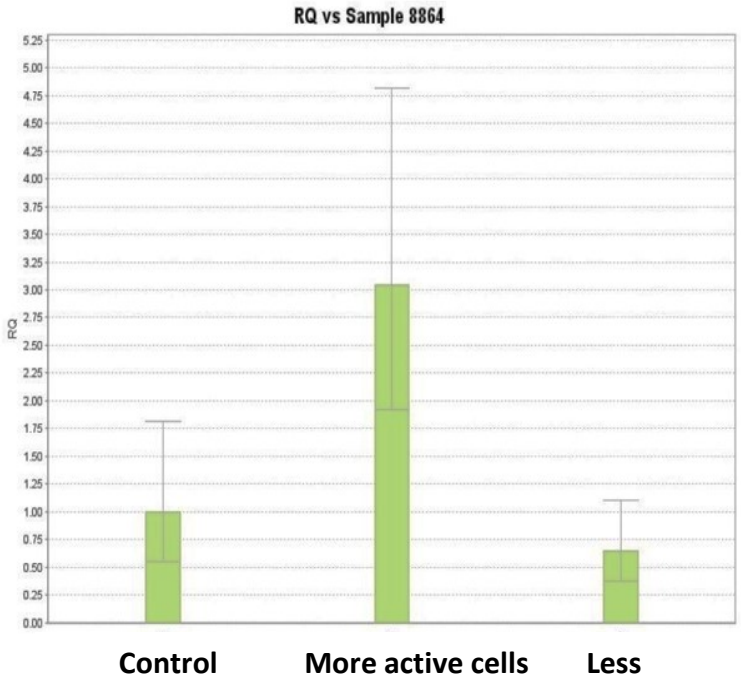

Figure 22: qPCR assay result; green bars represent ***result of qPCR t targeted assay*** *showing twice as much t variant DNA detected in the DNA sample extracted from cells with high level of MMP when compared to control (DNA extracted from heterozygous t/+ sample not sorted according to MMP).*

We were looking for enrichment of one sort of sperm cells according to t haplotype in tested groups. In affirmative case, there would have been found more of t sequence amplicon in one of tested DNA samples extracted form sorted group of cells. This would have been proven by stronger fluorescence signal.

Green bars represent result of fluorescent signal from t targeted qPCR assay. There is shown twice as much t variant DNA detected in the DNA sample extracted from cells with higher level of MMP than it is detected in control sample, which was extracted from cells of heterozygote t/+ not sorted according to MMP. As well, there is less DNA

detected in sample extracted from cells with lower MMP if we compare the signal to the control sample.

Since there was found stronger fluorescence signal in DNA extracted from more metabolically active cells, we could expect enrichment of t allele cells in this group. But, this result was attended by relatively big error bar and the result was not possible to conclude as significant. Therefore, the experiment was repeated on two more samples.

Based on this set of performed experiments, it was not possible to conclude any significant enrichment of the t allele bearing cells in any of the tested groups.

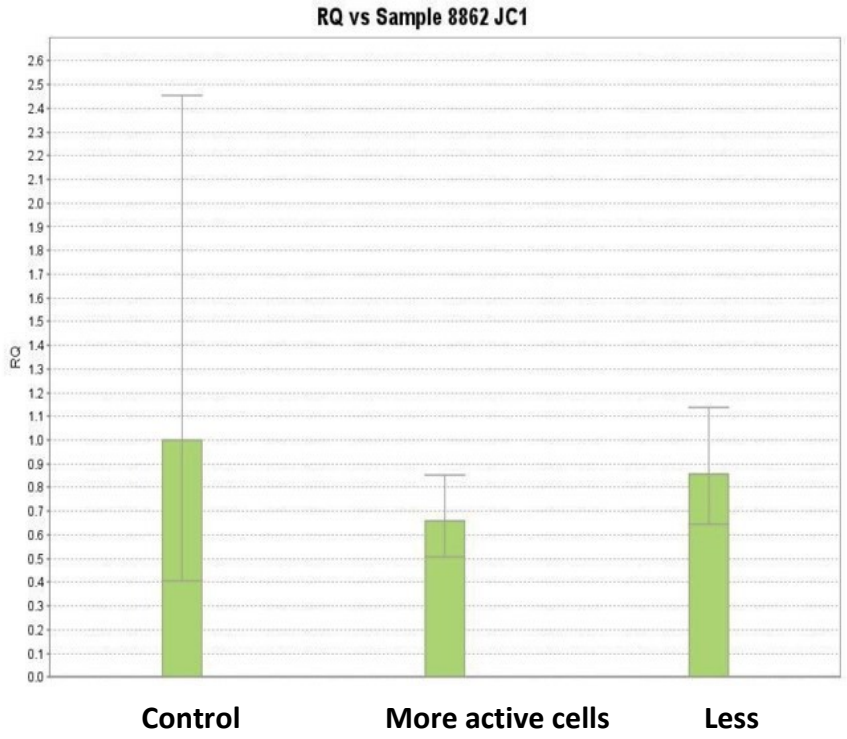

Figure 23: qPCR results; the size of error bar in control DNA sample does not allow us to conclude this experiment as determinant; no difference in DNA content observed.

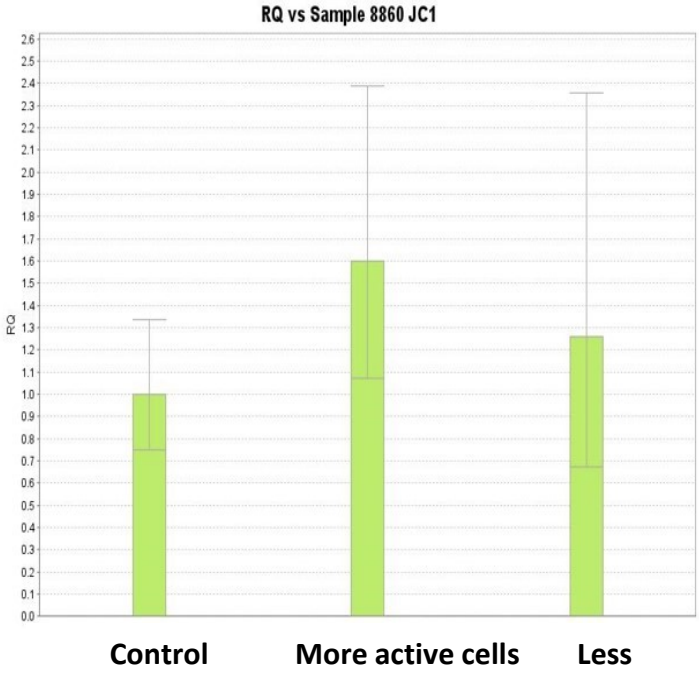

Figure 24: qPCR t targeted assay results; Fluorescent signal from DNA content of sorted samples was compared to DNA extracted from heterozygote t/+ not sorted according to MMP. Results do not show any significant difference in the amount of t variant DNA in these samples.

5. Discussion

As an interesting phenomenon, t-haplotype TRD has been attracting attention already for a long time. Preliminary knowledge about the genetic factors underlying this mechanism raises from classical genetic studies of last century (reviewed in Silver 1985). The current TRD model is based on molecular evidences using transgenic mice (reviewed in Herrmann and Bauer 2012). The only so far observed physiological evidence of expected mechanism is coming from comparison of sperm from homozygous t/t, heterozygous t/+ and wild type +/+ mice, stating that there are differences in motility and flagellar movement in between these genotypes (Olds-Clarke, 1996; Olds-Clarke & Peitz, 1985). Until recently, there was no more physiological data released.

According to our current belief, the TRD effect is brought about by motility differences between sperm carrying the t haplotype and wild type sperm from the same heterozygous male. This hypothesis was currently supported by results from Computer assisted sperm analyses (CASA) data, providing an information that there are indeed two subpopulations of sperm unequal in motility parameters possible to distinguish in heterozygous sample (Amaral, unpublished data). The concrete mechanism of this postulated sperm competition in favour of t haplotype bearing sperm remains to be clarified.

We have, for the first time, tested the hypothesis that the differences in sperm motility putatively responsible for transmission ratio distortion might be partly caused by metabolic cues.

Our theory, that energetic metabolism is in same way involved in TRD mechanism, was indeed supported by gathered data. Based on comparison of ATP levels in different genotypes of t, sperm ATP content was shown to be significantly lower in homozygous t/t samples. We have also shown that there are significant differences in mitochondrial functionality in between compared genotypes of t.

Our further intention was to examine the existence of sperm subpopulations in heterozygous t/+ sample not only in the matter of motility but also in the matter of energetic metabolism. Therefore, we tried to test the hypothesis, that subpopulations of sperm form heterozygous t/+ mice with higher MMP are also enriched in sperm of t or wt haplotype.

To do so, we designed an experiment which would support or disprove our hypothesis. We were working with heterozygous t/+ sample processed with JC-1 assay showing the quality of mitochondrial functionality. Sorting of a sperm subpopulation form such a sample enabled us to get a DNA extracted only from those sperm cells, which were characterised by high values (or low values) of MMP. A qPCR analysis of DNA from sorted subpopulations followed.

As it is shown in the results section, the original series of performed qPCR testing experiments evinced positive results and concluded properly working assays. The unpleasant change in efficiency of the method that appeared during the switch between optimization and real testing of sorted samples forced us to examine multiple criteria that might differentiate between optimizing runs and sorted samples analyses. Since we didn't find a cause of unspecific amplification in any of tested criteria, we focused on primer design itself.

The primer hb435-2 targeted on wild type region was excluded for its (later) obvious lack of specificity proved by strong band (in the same size of the main amplicon) in the electrophoresis result (Figure 17).

The reason why we didn't detect the deficient primer exclusivity in initial experiments, could be the amount of DNA inserted in the assay that provided peak of fluorescence signal in the melt curve strong enough to hide the second unwanted amplification. Even the last point of performed standard curve remained bigger in inserted DNA amount than the amounts that we can reach by DNA extraction from 100 000 sperm cells, which is the size of the sorted subpopulation. Although we did exclude the amount of entering DNA as a source of possible inhibition of the assay, it might be the reason why we didn't detect the primer incompetence in the beginning. As a second option remains an inhibition from unknown sources which was not present and affecting during the primer testing.

The very first future step, how to improve our experiment, would be to replace currently used primer couple by differently targeted one. By targeting the assay on different region in wt allele, we could try to reach the efficiency similar enough with t-targeted assay and prove each result by two assays, which was also our original goal when this experiment was designed.

Nevertheless, remaining t-targeted assay exhibited only low level of contamination (Figure 20) comparable with contamination observed in nontemplate control. Although explanation of the contamination is still missing, we believe, that the outcoming unidentified fluorescence differs from the level of the main amplicon (difference in Ct values) enough to start evaluating outcoming data.

The Mesogenin assay was added to the experiment to provide an information about amount of entering DNA. As such, the assay served as a control to the t-targeted assay. With this improvement, we were able to adjust the outcoming fluorescence signal to some minor DNA concentration distortions displayed by Mesogenin assay and observe results of t targeted assay.

Based on performed experiment, we believe that it is possible to observe a certain trend of correlation between MMP characteristic and haplotype of t. The so far outcoming ratios are suggesting a possible positive correlation between t-haplotype and high mitochondrial membrane potential in sperm. Such a result would support the theory that metabolic cues are at least partially responsible for the differences in sperm motility, which is putatively responsible for TRD.

Although we believe that the observed trend supports the theory of correlation between metabolic characteristic of the sperm cell and its genotype of t, without a confirmation by an improved experiment it is not possible to make any conclusion yet.

In the future, we would like to improve the experiment by adding third assay targeted to wt allele. With two testing assays and one control assay in the same qPCR plate, it would be possible to confirm each result in two ways by positive and negative result according to the target of the assay. We also believe, that the resolution of this method could reveal even more precise ratios of the t and wt allele contained in the tested sample. While testing on artificial prepared mixtures of homozygous t/t and +/+ DNA, we succeed with identification of the ratio 1:3. We also believe, that the resolution of the method has a future potential up to the ratio 1:5 identified in the artificial mixture and possibly even in a tested sample. Of course, an ability to recognize the ratio of alleles included in one sample with this resolution would bring a further dimension to our research.

At the moment, the working hypothesis that the differences in sperm motility are at least partly triggered by metabolic causes remains plausible. In the future, we would like to

comprehend if the motility patterns of t/+ and t/t sperm could be phenocopied in wild type +/+ sperm by certain metabolic shifts. Conceivable way how to do so would be incubating the wild type +/+ samples with known inhibitors of metabolic pathways and observing resulting motility parameters.

6. Summary

The mouse t-haplotype is the best studied example of transmission ratio distortion (TRD) in mammals. It is a selfish variant region on chromosome 17, transmitted as a unit in nonmendelian ratio (up to 99%) from heterozygous males to their offspring. This is believed to be caused by sperm motility differences between t allele carrying sperm and wt allele carrying sperm in heterozygous males. However, complete evidence for the postulated model is still missing. Here we test the hypothesis that the differences in motility are at least partly caused by metabolic cues and that the energetic metabolism is therefore involved in the mechanism of TRD.

In this study, we examined the possible differences in energetic metabolism between different genotypes of t (t/t, t/+, +/+). We proved that there are indeed statistically significant differences in the content of ATP and MMP values, by showing that the homozygote t/t has significantly lower values of both ATP and MMP when compared to the other two tested groups.

We also aimed to test the hypothesis, that subpopulation of sperm from heterozygote t/+ defined according to high MMP might be enriched in certain haplotype. Although we are not bringing a significant result, our preliminary data suggest, that this is indeed the case.

TRD is a genetic force, which affects allele frequency at the population. So far, it has been detected in many species including human. Although the prevalence of TRD in humans is not fully known, further investigation of this phenomenon has potential beneficial impact on many aspects of human genetics.

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